PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 45/05, 39/00, 37/48
A61K 37/62, C12N 9/02

A1

(11) International Publication Number: **WO 93/23080**

(43) International Publication Date: 25 November 1993 (25.11.93)

(21) International Application Number: PCT/US93/04582

(22) International Filing Date: 13 May 1993 (13.05.93)

(30) Priority data:
07/882,478 13 May 1992 (13.05.92) US

(71) Applicant *(for all designated States except US)*: THE BETH ISRAEL HOSPITAL ASSOCIATION [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : FOSSEL, Eric, T. [US/US]; 66 Priscilla Road, Chestnut Hill, MA (US).

(74) Agent: LORUSSO & LOUD; 440 Commercial Street, Boston, MA 02109 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: TARGETED ACTIVATED SPECIES CYTOTOXICITY

(57) Abstract

This invention comprises a method of treating animals, including humans, for conditions such as cancer by producing discrete site cytotoxic environment in an animal, including a human, by the steps of administering to the animal a therapeutically effective dosage of a prooxygenator-affixation element complex; in conjunction with, administering to the animal a therapeutically effective amount of an oxygen source substrate thus producing oxygen free radical species including superoxide at the site of binding. The present invention further comprises a prooxygenator-affixation element complex. In one embodiment the prooxygenator aspect is xanthine oxidase, the affixation element is a tumor specific antibody and the oxygen source substrate is xanthine.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

TARGETED ACTIVATED SPECIES CYTOTOXICITY

Field of the Invention.

This invention comprises a method of treating animals, including humans, for conditions such as cancer by producing a discrete site cytotoxic environment in an animal, including a human, by the steps of administering to an animal a therapeutically effective dosage of a proöxygenator-affixation element complex; in conjunction with, administering to the animal a therapeutically effective amount of an oxygen source substrate thus producing oxygen free radical species including superoxides at a cytotoxic level at the site of complex binding. The present invention further comprises a proöxygenator-affixation element complex. In one embodiment the proöxygenator moiety is xanthine oxidase, the affixation element is a tumor specific antibody and the oxygen source substrate is xanthine.

Background of the Invention.

The earliest medicinal agents were administered either typically or by ingestion with little control over the site of drug action. The discovery of penicillin brought the "magic bullet" to the practice of medicine. Since then pharmacology has continued to refine techniques to bring the active agents into the closest proximity with the site of action. For example,

today, radio labeled antibodies are used to localize sites in diagnostic procedures. Similarly, IL-2 binding sites have been linked to diphtheria toxin to target and destroy activated T-cells. However, this last approach has been limited to cell by cell killing of those cells which actually phagocytize the diphtheria toxin molecule.

A major problem with chemotherapy is toxicity. Chemotherapeutic agents are characterized by high toxicity. This toxicity is only slightly discriminatory, and, as a general principal, attacks the entire body injuring or destroying both normal and abnormal tissue. Many solid tumors are well vascularized. However cellular antitumor agents have difficulty reaching tumor cells, in part, due to a fibrin barrier. In certain instances, the fibrin barrier may be eliminated by thrombolytic agents. In other instances, treatment of cancers, and particularly solid tumors, is hampered by inadequate circulatory investment of such tumors. In fact, the most rapidly growing tumors may be the most difficult ones in which to obtain therapeutic concentrations of anti-tumor agents.

The present invention is, in its preferred embodiment, directed to providing the delivery of one or more therapeutic agents quite specifically to a given site, but not so specifically that only a given individual cell is treated. The therapeutic agents are activated oxygen species (collectively,

"AOS"). AOS of the present invention include peroxides such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), and singlet oxygen ($^1O_2$). It is just such AOS that have been conjectured as active agents in polymorphoneuclear neutrophils after activation by pathogens, cytokines or other cell activators.

This invention utilizes the known technology of binding compounds to antibodies so that neither the ability of the antibody to bind to antigen nor the activity of the bound compound is impaired. An examples of this technology are U.S. Pat. No. 4,671,958 issued to Rodwell et al., and U.S. Pat. No. 4,867,973 to Goers et al, the teachings of each being incorporated herein by reference. U.S. Pat. No. 4,671,958 describes a method for site specific covalent attachment of a compound to an antibody molecule by selectively oxidizing a carbohydrate moiety of the antibody, located outside the antigen binding region of the antibody, to form an aldehyde group with an amine group (such as a primary amine, secondary amine, hydrazine, hydrazide, hydroxylamine, phenylhydrazine or semicarbazide) to form a Shiff base (e.g., oxime, hydrazone, phenylhydrazone, or semicarbazone, respectively).

Accordingly, substrate linkers are modified by attaching hydrazine or hydrazide derivatives to one end of the linker. The unmodified sites on the linker may or may not be covalently attached to a compound. Linkers are synthetic or naturally

occurring substrates which are susceptible to cleavage by any of the components of complement. A number of such linkers are described and disclosed in U.S. Pat. No. 4,671,958, including N-Boc-tyrosine o-nitrophenyl ester, N-acetyl-gly-lys-methyl ester and others well known in the art.

By way of example, substrate linkers which are attached to a compound via an ester or amide link, are modified by attaching a hydrazide such as phenylhydrazine to the opposite amino terminus of the peptide chain. The hydrazide derivative of the peptide linker is attached to a compound via an ester or amide link is then reacted with an oxidized immunogloublin fragment containing an oxidized carbohydrate. This results in hydrazone formation and the covalent attachment of the compound to the carbohydrate side chain of the immunoglobulin via a linker group which is susceptible to cleavage by complement. The described covalent attachment of linker to the carrier antibody does not interfere with the antigen binding site of the molecule nor with complement fixation. Schematically this may be represented:

$$\text{antibody-}\left\{\begin{matrix}\text{carbohydrate}\\ \text{side-chain}\end{matrix}\right\}\text{-CH=N-NH- -NH-}_{\text{linker}}\text{-[ß]-compound}$$

where ß represents an amide or ester bond.

Summary of the Invention.

This invention includes a proöxygenator-affixation element complex. In particular embodiments the proöxygenator-affixation element complex comprises a proöxygenator moiety of at least one

enzyme, such as xanthine oxidase, superoxide dismutase, or a myeloperoxidase. In specific embodiments of the invention the proöxygenator-affixation element complex comprises an affixation element being an antibody. Particular antibodies are those that bind to melanoma, carcinoma, adenocarcinoma, sarcoma, neuroblastoma, myeloma, lymphoma, or leukemia cells. Examples within the invention are antibodies such as α-MSH, carcino-embryonic antigen, α-fetoprotein, or SSEA-1. Other examples are wherein proöxygenator-affixation element complex comprises an affixation element being an peptide such as the diphtheria fragment B, or IL-2 binding site.

This invention also includes a method of producing discrete site cytotoxic environment in an animal, including a human, comprising the steps of:

(i) administering to said animal a therapeutically effective dosage of a proöxygenator-affixation element complex wherein said complex has a binding affinity for the site of cytotoxic environment production; and thereafter,

(ii) administering to said animal a therapeutically effective amount of an oxygen source substrate. In a particular embodiment, upon administration, the affixation element of the proöxygenator-affixation element complex performs the step of binding the complex to a cell. Some proöxygenator elements of the method are xanthine oxidase, superoxide dismutase, or

myeloperoxidase. Certain oxygen source substrates of the method are methylxanthines such as xanthine, caffeine or theophylline. The method of this invention also encompasses the step of maintaining the AOS concentration in a discrete area to at least about 10^{-8} M/minute for a particular intervals of at least about 15 minutes, and preferably at least about 10^{-6} M/minute for a particular interval or intervals of at least about 15 minutes, and more preferably at least about 10^{-5} M/minute for a particular intervals of at least about 15 minutes. In particular aspects the method includes administering to an animal a therapeutically effective dosage of a proöxygenator-affixation element complex which comprises binding said complex to at least about 50% and preferably at least about 80% of the binding sites at the general site of cytotoxic environment production.

In a diagnostic application, this invention includes adding to a tissue culture of a tumor to be tested two or more graduated dosages of a proöxygenator-affixation element complex wherein said complex has a binding affinity for the tumor being tested; and thereafter,

administering to said culture a therapeutically effective amount of an oxygen source substrate;

determining tumor growth inhibition in said tissue culture.

Detailed Description of the Invention.

This invention will best be understood with reference to the following definitions:

A. Proöxygenator shall mean at least one moiety which produces an AOS upon exposure to at least one oxygen bearing substrate.

In some applications it will be appreciated that multiple proöxygenator moieties may be attached to a single affixation moiety. These may be the same proöxygenator moieties or different proöxygenator moieties. In one example, xanthine oxidase and a peroxidase such as superoxide dismutase may be cojoined to a single affixation moiety. Additionally, marker moieties such as radio labels, fluorescent materials or NMR labels may be affixed.

B. AOS of the present invention shall mean activated oxygen species including peroxides such as hydrogen peroxide (H_2O_2) and oxygen free radicals, (O_2^-), HO·, and HOO·. The particular AOS, O_2^-, is termed "superoxide."

AOS of this invention further include singlet oxygen ($^1O_2$).

Paradigm reactions of this invention are (1) the conversion of xanthine to superoxide, the oxygen free radical (O_2^-) by the enzyme xanthine oxidase, and (2) the conversion of xanthine to uric acid and superoxide, an oxygen free radical (O_2^-).

Without being bound to a particular theory, it is believed that the efficacy of this invention is a consequence of the provision of AOS in therapeutically effective concentrations to the site of complex binding. While not bound by any particular scheme by which the provision of AOS to the site of complex binding provide therapeutic efficacy, it is believed that the desired reaction such as tumor toxicity is substantially similar to the cytotoxic and bactericidal system found in polymorphoneuclear neutrophil leukocyte (PMN). In PMN systems, researchers have found that (O_2^-), HO·, and HOO· are directly cytotoxic. In addition H_2O_2 may react with Cl^- to form OCl^- (hypoclorite ion) which is a bactericidal agent. In addition to hydrogen peroxide, the oxygen radical, singlet oxygen (O_2^-), and hydroxy radical (HO·) are also associated with bactericidal/anti-pathogen activity. In a similar fashion, macrophages taken from BCG-infected animals or otherwise activated have been reported as destroying tumor cells in tissue culture through elaboration of hydrogen peroxide and tumor necrosis factor.

C. Affixation element shall mean a cell receptor site moiety such as an antibody or peptide capable of affixing the complex to a site on a cell. The affixation element of the complex is understood to have a binding affinity for the site of cytotoxic environment production. This can be at a site

particular to a tumor, but also particular to certain classes of cells such as interleukin binding cites. An affixation element will also be required to cojoin at least one proöxygenator moiety and preferably more than one such moiety. Examples of affixation elements are the cell binding fragment of diphtheria toxin (fragment B), the IL-2 binding site, and antitumor antibodies such as α-MSH.

It is understood that in the practice of this invention, some sites undergo phagocytosis. That is the site of cellular affixation which is initially external becomes drawn into the cell. While it is preferred that the cell bound proöxygenator-affixation element complex remain external to the cell, this is not an absolute requirement. Antibodies are a particular category of affixation element, generally comprising proteins circulating in plasma.

D. Complex shall mean a proöxygenator moiety bound to an affixation element such that (1) the proöxygenator moiety remains capable of enzymatically converting an oxygen source substrate into AOS, and (2) the affixation element as complexed to the proöxygenator moiety maintains specificity for the target site of affixation.

E. Xanthine oxidase shall mean the the enzyme xanthine:oxygen oxidoreductase, an iron-molybdenum flavoprotein.

F. Discrete site cytotoxic environment shall mean the provision of a cytotoxic environment at a defined location proximate to a proöxygenator-affixation element complex bound to a cell, but not limited to the single bound cell.

G. Cytotoxic environment shall mean an environment that results in reduction or cessation of proliferation of a cell type and further may include death of some or all cells of a given cell type. Cell is used as an inclusive term encompassing differentiated tissue, single cells, bacteria, multicellular pathogenic organisms, viri, retroviri, and neoplastic cells.

Cytotoxic environment shall further be expansively understood to include AOS as a "neo-adjuvant," that is as a potentiator of other therapies. The neo-adjuvant function is displayed in conjunction with other therapy such as radiation, chemotherapy, and vaccine/immunomodulation therapy -- each of which is potentiated by cellular changes including permeability changes and protein expression/recognition changes resulting from the practice of this invention.

H. Tumor specific antibody shall mean an antibody that preferentially binds to neoplastic cells. In particular embodiments, antibodies to malignant melanoma, carcinoma, adenocarcinoma, sarcoma (including, Kaposis sarcoma), neuroblastoma, myeloma, lymphoma, and leukæmias.

I. Xanthine shall refer to methylxanthines and analogues and derivatives thereof. This shall be understood to include, without limitation, hypoxanthine, caffeine, theophylline, theobromine, dysphylline, enprofyline, and pentoxifylline.

J. Therapeutically effective shall mean a dosage that produces the desired physiological effect. As to a proöxygenator-affixation element complex, therapeutically effective means that sufficient complex is bound such that when presented with oxygen bearing substrate a cytotoxic environment arises. In the practice of the method of this invention two steps are required. First the complex must be bound to the target cells in therapeutically effective concentration -- which is necessarily a potential for physiological activity realized as permanent effect only upon the presentation of oxygen bearing substrate. Therapeutically effective as to a dosage of oxygen bearing substrate shall be one sufficient to establish a cytotoxic environment at the site of complex binding in the presence of bound complex. Such dosage provides an environment that results in reduction or cessation of proliferation of a cell type and further may include death of some or all cells of a given cell type or at a given location.

In the practice of this invention it will be of importance to select an affixation element that will bind to a target cell in sufficient concentration to ultimately provide therapeutically

effective AOS concentration at the target site. While some pathogens are exquisitely sensitive to AOS others are recalcitrant. Binding of complex at a high density of sites at a high saturation for a lengthy period will be factors tending to increase obtainable AOS levels. Other factors are the number of proöxygenator moieties bound to each antibody, the activity of each proöxygenator moiety, and availability of AOS substrate and the absence of competitive or inhibitory reactants.

Tumor Specific Antigens: Tumor cells can frequently be targeted by antigenic determinants. Cells infected with oncogenic viri frequently have two recognition antigens displayed on the cell surface, either of which may provide suitable sites for antibody binding. Oncofetal antigens may be expressed on the surface tumor cells which differentiate adult tissues from tumor tissues. Examples of these are carcino-embryonic antigen (CEA) in cancer of the intestine and α-fetoprotein in hepatic carcinoma. There are available monoclonal antibodies raised against human melanoma cells that also react with tumors of neural origin. Another monoclonal antibody defines the SSEA-1 antigen found on a variety of human tumors. Tumors induced by chemical agents such as benzopyrene have tumor specific antigens. Researchers have particularly noted the tumor specificity of the

Ig idiotype on the surface of chronic leukæmic cells. Other tumor specific antigens can be prepared by methods well known in the art and do not comprise a part of this invention.

Tumors sensitive to the AOS therapy of this invention, and therapeutically effective dosage levels may be determined by *in vitro* techniques which are known in the art. For example, a tumor may be conveniently grown in tissue cultures. To the tissue cultures a variety of proöxygenator-affixation element complexes at a variety of concentrations may be presented with various oxygen source substrates in a checker board assay or the like. The most inhibited tissue cultures will define the therapeutically effective complexes, oxygen source substrates, and may be extrapolated to define a range of therapeutically effective dosages. Additional agents may be cross tested in, for example, traditional *in vitro* Combination Effect Test or the Therapeutic Index Test, to determine if neo-adjuvant activity may be advantageously used as well.

The Combination Effect Test employs a series of tests to determined combined drug efficacy. One such test is the "Checker Board Assay" to test different serial dilutions of the drugs to be combined with AOS administration as challenged by a test cell culture of cancer cells in agar or broth. Another test is the Virus Titer Reduction Assay, measuring the reduction in multiplication of virus as grown in host cells. Another test is

an increase in the therapeutic index which is the dose lethal to 50% of the subjects as compared to the dose therapeutically effective in 50% of the cases. The use of the Combination Effect Test allows for the coädministration of AOS with other drugs in a useful and efficacious manner. Particular reference is made to the increased efficacy of Tumor Necrosis Factor by the practice of this invention.

Complexing a Proöxygenator with an Affixation Element: In combining a proöxygenator moiety with an affixation element care must be taken to preserve the AOS forming activity (usually enzymatic) of the proöxygenator and the binding strength and specificity of the affixation element. To accomplish complexing either chemical or recombinant methods may be usefully employed. As a cojoining methodology, hybridizing IL-2 with a toxin has been described in Greenfield et al., "Science," pp 238, 536 (1979). Also, hybridization of diphtheria toxin/IL-2 has been described in U.S. Pat. No. 4,675,382 using recombinant DNA methodologies. Pseudomonas exotoxin A/IL-2 hybridization has been described in Lorberboum-Galski et al., "Proc. Natl. Acad. Sci. USA 85: 1922-26, (1988). The teachings of the foregoing references are incorporated herein by reference. In, for example, Lorberboum-Galski et al., IL-2 replaced the endogenous cell-specific receptor domain of the toxin protein, Pseudomonas exotoxin A/IL-2. Further examples of this technology are set

forth in US Patent 5,047,227 to Rodwell, "Novel and Improved Antibodies for Site Specific Attachment of Compounds;" US Patent 4.937,183 to Ultee et al., "Method for Preparation of Antibody-fragment Conjugates;" US Patent 4,867,973 to Goers et al., "Antibody-Therapeutic Agent Conjugates;" and US Patent 4,671,958 to Rodwell et al., "Antibody Conjugates for the Delivery of Compounds to Target Sites" the teachings of which are incorporated herein by reference. Similar information is setforth in European Patent Application 90311590.5, Publication No. 425,235 A2, by Chari et al. the teachings of which are incorporated herein by reference.

The compositions and methods of this invention possess valuable pharmacological properties. The proöxygenator-affixation element complex can localize on or near such targets as tumors cells, cysts, areas of inflammation, and individual viri or retroviri. In the presence of an oxygen source substrate, the proöxygenator-affixation element complex will provide discrete site cytotoxic environment. Such discrete site cytotoxic environment will retard or reverse growth of the target cells or organisms. In some applications the desired effect will further include cytotoxic treatment of other nearby cells or organisms at the same discrete site. The discrete site cytotoxic effect is of great benefit in the field of medicine, particularly in the field of cancer therapy. This benefit is demonstrated, for example,

using the method of administering a complex of tumor specific antibody-xanthine oxidase in conjunction with administration of xanthine. A cytotoxic environment at the tumor site is established to preferentially kill tumor cells, with minimal off site toxicity.

Thus, these compositions can be used with indications providing a binding site for the complex. Included indications are solid tumor neoplasms as well as systemic neoplasms including cancers, leukæmias, viral diseases wherein the virus is "recognized" and attached by the antibody, brucellosis, shistomiasis, malaria, and bacterial infections.

The compositions and method are particularly useful as antitumor agents wherein the tumor is strongly antigenically identifiable by the antibody of the complex and wherein the tumor is susceptible to AOS. The composition can be used in conjunction with other therapeutic agents as a neo-adjuvant.

In addition, the compositions can be used in *in vitro* diagnostics for determining which target cells are sensitive or susceptible to treatment via AOS (alone or in combination with other drugs) at concentrations obtainable *in vivo*.

The compositions of this invention are generally administered to animals, including but not limited to mammals, and avians, and particularly, livestock, household pets, humans, cattle, cats, dogs, poultry, etc.

The pharmacologically active compositions of this invention can be processed in accordance with conventional methods of Galenic pharmacy to produce medicinal agents for administration to patients, e.g., mammals including humans.

The compositions of this invention can be employed in admixture with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral (e.g., oral or inhalation) or topical application which do not deleteriously react with the active compositions. Suitable pharmaceutically acceptable carriers include but are not limited to water, and salt solutions (e.g., isotonic saline, buffered saline) and injectable formulations (including i.v., and peritoneal) .

The pharmaceutical preparations can be sterilized but must not be denatured. If desired, pharmaceutical preparations may be mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, and the like which do not deleteriously react with the active compositions. They can also be combined where desired with other active agents, e.g., proöxygenator-affixation element complex administered with an oxygen source substrate.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably aqueous solutions, as well as suspensions, or emulsions. Ampoules are convenient unit dosages. In certain localized administrations the proöxygenator-affixation element complex and/or oxygen source substrate may be administered via intravenous shunt permitting "up stream" introduction of therapeutic agents and "down stream" removal of therapeutic agents. Thus, high localized concentrations of therapeutic agents may be obtained, and yet maintain low systemic levels.

Sustained or directed release compositions can be formulated, e.g., liposomes, or those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc. It is also possible in certain applications to freeze-dry the new compositions and use the lyophilates obtained, for example, for the preparation of products for injection.

For topical application such as to the lungs, suitable are sprayable aerosol preparations wherein the active ingredient, preferably in combination with a liquid inert carrier material, is packaged in a squeeze bottle or provided by nebulizer.

Intravenous administration is preferred. However, the specific mode of administration will vary with the site of treatment and the particular active agents. The method of administration will preferably be selected to develop the highest AOS concentration at the site of treatment.

Dosages of both the proöxygenator-affixation element complex administered and the oxygen source substrate(s) may be determined empirically by methods known to those skilled in the art. However the method and agents of the instant invention are uniquely determinable by calculation. An antibody's affinity for target binding sites is determinable by standard methods. Similarly, the general number of binding sites in a given antibody-receptor application of the invention is determinable.

In the case of superoxide (O_2^-) as produced by xanthine oxidase, the following calculations are instructive.

1. Each xanthine throws off one superoxide, O_2^-.
2. The specific activity of xanthine oxidase is ≈ 14,000, thus the enzyme can produce 14,000 μM of O_2^- per minute.
3. A given cell has about 40,000 binding sites for a given antibody.
4. Based on a single cell (and presuming only one enzyme per antibody), the area local to that cell may have 5.6×10^8 μM O_2^-/min, or roughly 560M/sec.
5. The lifetime of superoxide is about 10^{-6} to 10^{-9}.

6. Thus maintained site concentration at an instantaneous sampling is between about 10^{-5} to about 10^{-8} M of superoxide/minute.

Concentration levels can be altered by binding more than one enzyme to an antibody, or utilizing enzymes of increased activity. Further, attachment of antibody and associated enzymatic activity as generally distributed in an area will result in nodes of increased AOS concentration.

While these will vary widely with each antibody, binding site, volume over which antibody complex is distributed and the half-life of the complex, such determinations are within the recognized skill of practitioners in the art. Dosages based on these factors -- bearing in mind tolerable toxicity levels -- will then be determined.

In a like fashion, the dosage and time of administration of oxygen source substrate(s) to form AOS from a complex containing xanthine oxidase may be either determined empirically or calculated. In the example of the methylxanthine, caffeine, as an oxygen source substrate(s), the dosage of caffeine will not exceed the capacity of the xanthine oxidase to form AOS. Calculation will include volume throughout which the xanthine is distributed and the half-life of caffeine *in vivo*. In the example of caffeine and theophylline, in humans it is known to be distributed into all body compartments, and its apparent

distribution is about 0.4 to about 0.6 liter/kg of body weight, and higher in premature infants. The half-life of caffeine in plasma is about 3 to 7 hours. Variance in the half-life, however, in specific circumstances is well known to those skilled in the art. For example, the half-life may double in women in the later stages of pregnancy, or be up to 50 hours in premature infants. There is also well document substantial inter-individual variation in clearance of methylxanthines, and such clearance should be tested to determine the individual dosage requirements. Caffeine dosages typically should not exceed 15 mg/kg and plasma concentrations of 30μg/ml. Tolerated methylxanthine dosage levels are well known in the art, such as are found in Goodman and Gilman's The Pharmacological Basis of Therapeutics Eighth Edition, Eds., Gilman, Rall, Nies, Taylor (Pergamon Press, New York, New York, 1990), the teachings of which are incorporated herein by reference.

The dosage of the compositions according to this invention generally are designed to afford maximal tolerated delivery of AOS to the target site. It will be appreciated that the actual preferred amounts of active compositions in a specific case will vary according to the specific compositions being utilized, the particular compositions formulated, the mode of application, and the particular situs and organism being treated. Dosages for a given host can be determined using conventional considerations,

e.g., by customary comparison of the differential activities of the subject compositions and of a known agent, e.g., by means of an appropriate, conventional pharmacological protocol.

In the practice of this invention utilizing xanthine oxidase bound to antibody the proöxygenator-affixation element complex the following steps are taken. A subject in need of AOS therapeutic treatment and having an antibody specific treatment site is administered xanthine to a concentration of about 10^{-9} to about 10^{-5} M. Particular effective concentrations are from about concentration of about 10^{-8} to about 10^{-6} M, as well as from about concentration of about 10^{-6} to about 10^{-5} M. If toxicity is at issue, maximum concentration is established over time, with xanthine administration curtailed when unsuitable toxicity begins to be manifested. Maximum concentration is reached about 1 hour after oral administration. In a 70kg subject, administration of xanthine in doses of from about 300 mg to 500mg is useful. Thereafter the proöxygenator-affixation element complex, xanthine oxidase bound to an antibody specific to the treatment site, administered intravenously to establish a concentration which will bind to binding sites in from about 20% to 100% of such sites. Xanthine oxidase bound to said antibody is periodically readministered in proportion to the rate at which enzyme-antibody is deactivated, here about every three hours. Due to the long half-life of xanthine, it is not usually necessary to

readminister xanthine during the course of this treatment. In particular embodiments it is useful to administer the proöxygenator-affixation element complex prior to administration of the substrate.

Example 1

Xanthine Oxidase/α-MSH Complex

To a human suffering from malignant melanoma, xanthine is administered intravenously to obtain a plasma level of 10-30μg/ml which is maintained over 4 hours by additional xanthine administration as required. Twenty minutes after initial xanthine administration, a proöxygenator-affixation element complex consisting of a proöxygenator moiety of xanthine oxidase and an affixation element of α-melanocyte stimulating hormone (α-MSH) is administered, i.v. The xanthine oxidase/(α-MSH complex is suspended in isotonic saline. Administration is intravenous at a dosage of 100 mg every ten minutes until 80% of the binding sites on target cells are occupied. As used herein binding cites on target cells refers to the binding of the proöxygenator-affixation element complex the at the site of cytotoxic environment production. Such binding results from the affinity between complex and binding cite. This treatment is repeated daily for 5 days.

I claim:

1. A proöxygenator-affixation element complex.

2. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises a proöxygenator moiety of at least one enzyme.

3. The complex of Claim 2 wherein the enzyme is a xanthine oxidase.

4. The complex of Claim 2 wherein the enzyme is a superoxide dismutase.

5. The complex of Claim 2 wherein the enzyme is a myeloperoxidase.

6. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises an affixation element being an antibody.

7. The complex of claim 6 wherein the antibody binds to melanoma, carcinoma, adenocarcinoma, sarcoma, neuroblastoma, myeloma, lymphoma, or leukemia cells.

8. The complex of Claim 6 wherein antibody is α-MSH, carcino-embryonic antigen, α-fetoprotein, or SSEA-1.

9. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises an affixation element being an peptide.

10. The complex of claim 9 wherein the peptide is the diphtheria fragment B, or IL-2 binding site.

11. A method of producing discrete site cytotoxic environment in an animal, including a human, comprising the steps of

administering to said animal a therapeutically effective dosage of a proöxygenator-affixation element complex wherein said complex has a binding affinity for the site of cytotoxic environment production; and thereafter,

administering to said animal a therapeutically effective amount of an oxygen source substrate;

forming an activated oxygen species (collectively, "AOS").

12. The method of Claim 11 wherein upon administration said affixation element of the proöxygenator-affixation element complex performs the step of

binding the complex to a cell.

13. The method of Claim 11 wherein the proöxygenator element comprises xanthine oxidase, superoxide dismutase, or myeloperoxidase.

14. The method of Claim 11 wherein the oxygen source substrate is a methylxanthine.

15. The method of Claim 14 wherein the methylxanthine is xanthine.

16. The method of Claim 14 wherein the methylxanthine is caffeine.

17. The method of Claim 14 wherein the methylxanthine is theophylline.

18. The method of Claim 11 further comprising the step of maintaining the AOS concentration in a discrete area to at least about 10^{-8} M/minute for a particular intervals of at least about 15 minutes.

19. The method of Claim 18 further comprising the step of maintaining the AOS concentration in a discrete area to at least about 10^{-6} M/minute for a particular intervals of at least about 15 minutes.

20. The method of Claim 19 further comprising the step of maintaining the AOS concentration in a discrete area to at least about 10^{-5} M/minute for a particular intervals of at least about 15 minutes.

21. A method of Claim 11 wherein

administering to said animal a therapeutically effective dosage of a proöxygenator-affixation element complex comprises binding said complex to at least about 50% of the binding cites at said site of cytotoxic environment production.

22. The method of Claim 21 wherein said binding is to at least about 80%.

23. A method of diagnosing AOS treatable tumors comprising:

adding to a tissue culture of a tumor to be tested two or more graduated dosages of a proöxygenator-affixation element complex wherein said complex has a binding affinity for the tumor being tested; and thereafter,

administering to said culture a therapeutically effective amount of an oxygen source substrate;

determining tumor growth inhibition in said tissue culture.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04582

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 45/05, 39/00, 37/48, 37/62; C12N 9/02
US CL :424/94.2, 94.1, 94.3, 85.1, 85.2, 85.91; 435/189
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.2, 94.1, 94.3, 85.1, 85.2, 85.91; 435/189; 514/410, 185

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, Medline, Biosis, Registry

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,906,469 (Jansen, et al.) 06 March 1990, see entire document, especially col. 1, lines 26-31, col. 5, lines 11-12, col.6, lines 6-8 and 54-64, and col. 13, lines 6-9.	1-23
Y	MOLECULAR AND CELLULAR BIOCHEMISTRY, Vol. 10(1), issued 31 January 1976, A. Bozzi, et al., "Enzyme Defense Against Reactive Oxygen Derivatives. II. Erythrocytes and Tumor Cells," pages 11-16, especially pages 11 and 12.	1-23
Y	US, A, 4,971,991 (Umemura, et al.) 20 November 1990, see entire document.	1-23
Y	US, A, 4,975,278 (Senter, et al.) 04 December 1990, see entire document.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 JULY 1993	29 JUL 1993

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	KRISTIN K. LARSON
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.
PCT/US93/04582

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,762,707 (Jansen, et al.) 09 August 1988, see entire document.	1-23
A	US, A, 4,937,183 (Ultee, et al.) 26 June 1990.	1-23
A	US, A, 4,671,958 (Rodwell, et al.) 09 June 1987.	1-23
A	US, A, 4,867,973 (Goers, et al.) 19 September 1989.	1-23
A	ACCOUNTS OF CHEMICAL RESEARCH, Vol. 5(10), issued October 1972, I. Fridovich, "Superoxide Radical and Superoxide Dismutase," pages 321-326.	1-23